Genes encoding endo-ß-mannanases in *Brachypodium distachyon* seeds have a role not only in cell wall softening upon germination *sensu stricto* but also during post-germination reserve mobilization

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Brachypodium distachyon seeds are characterized by presenting low levels of starch and high levels of (1,3; 1,4) β-glucanns in its endosperm cells besides having thick mannan-rich cell walls that confer a strong mechanical resistance. Endo-β–mannanases (MAN; EC. 3.2.1.78) are hydrolytic enzymes that catalyze cleavage of β1→4 bonds in the mannan-polymer.

In the genome of Brachypodium, the *MAN* family is represented by six members (Fig. 1A, B).

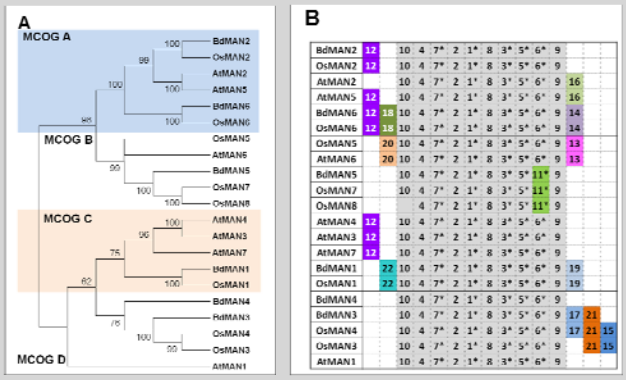

Figure 1: A Phylogenetic tree with deduced protein MAN sequences of Brachypodium, *Oryza sativa* and *Arabidopsis thaliana*; bootstrapping values are indicated in the branches. **B** Schematic distribution of conserved motives among the deduced protein sequences in **A**, that were identified by means of the MEME software.

We have systematically explored the expression of the six *MAN* genes in different organs (leaves, roots, spikes, developing and germinating seeds). The *BdMAN3* is the most highly expressed gene in the germinating seeds, and its expression decreases upon germination in aleurone. However, *BdMAN2* and *BdMAN6* are highly induced during this process in embryos reaching a maximum at 36 hours of water imbibition (Figure 2).

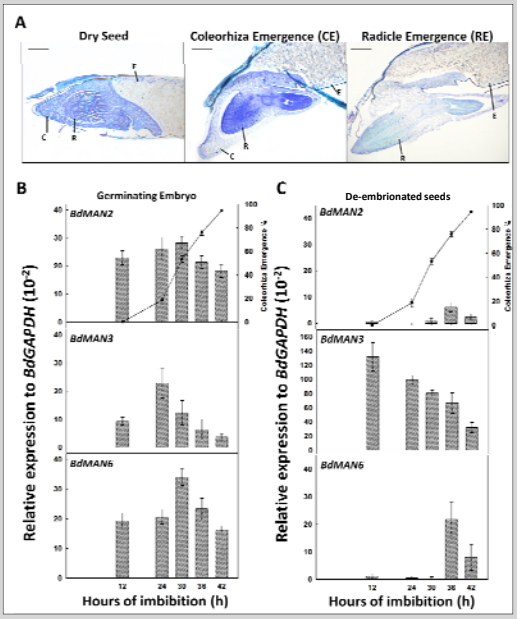

Figure 2: A *Brachypodium distachyon* dry and germinating seed sections stained with toluidine blue. **B** Transcript analysis by RT-qPCR of the three mannanase genes expressed during the germination of *B. distachyon* seeds (*BdMAN2*, *BdMAN3 and BdMAN6*) at different hours of imbibition (h). Error bars indicate standard deviations of three independent experiments. *Brachypodium* seeds reach 100% germination (coleorhiza emergence) at 42 h in the conditions of the assay: 23ºC, in darkness.

The *BdMAN2* and *BdMAN6* transcripts accumulate in the coleorhiza while those of *BdMAN3* appear mainly in the radicle tip (Figure 3).

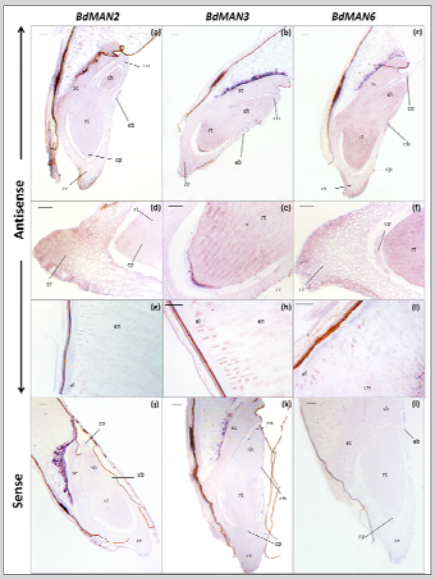

Figure 3: *In situ* mRNA hybridization analyses of *BdMAN2*, *BDMAN3* and *BdMAN6* in 27 h imbibed seeds. *BdMAN2* (**a, d, g, j**), *BdMAN3* (**b, e, h, k**) and *BdMAN6* (**c, f, i, l**). Longitudinal sections of **Germinating seeds** (**a, b, c, j, k, l**). Close-up of the coleorhiza in **a, b, c (d, e, f)**. Close-up of the aleurone in **a, b, c (g, h, i)**. *al*: aleurone layer; *cp*: caliptra; *co*: coleoptyle; *cr*: coleorhiza; *en*: endosperm; *ep*: epyblast; *rt*: root; *sc*: scutellum; *sh*: shoot.

Mannan polymers *in situ* immunofluorescence analyses have been carried out in partial longitudinal sections of *B. distachyon* imbibed seeds, and the LM21 antibody that specifically recognizes mannan, glucomannan, and galactomannan polysaccharides has been used. At 12 h of imbibition, mannan polymers are mainly localized to the coleorhiza (c) and the root tips (R; Figs. 4A-C). Interestingly, heteromannans are barely detected in a later germination phase (at 27 h), suggesting an embryo cell wall dynamics during seed imbibition (Figure 4E-D).

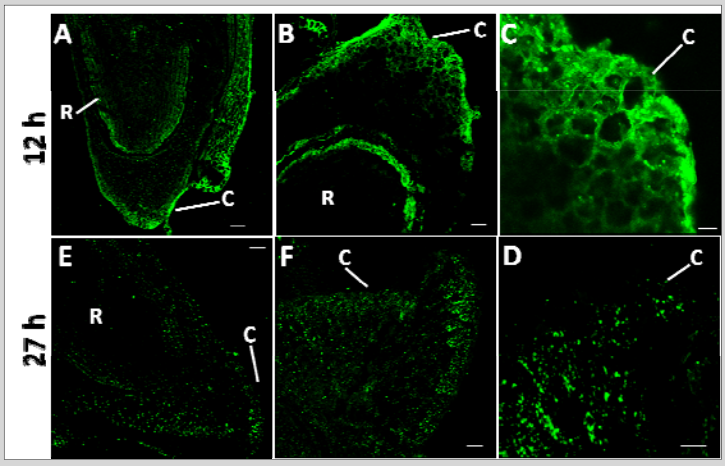

Figure 4: Mannan polymer immunolocalization in longitudinal sections of Brachypodium germinating seeds at 12 h (**A**, **B**, **C**) and at 27 h (**E**, **F**, **G**). R: Radicle; C: Coleorhiza.

Our results indicate that *BdMAN* genes are implicated not only in the weakening of the coleorhiza during seed germination as occurs with *AtMAN7* and *AtMAN6* in *Arabidopsis thaliana* (1, 2, 3), but their *mRNA* tissue localization to the aleurone layer (Figure 3) suggests a role in post-germination reserve mobilization (ms in preparation).

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This work was financially supported by : **BFU2009-11809 (i.p.: Prof. P. Carbonero)**

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P08-17

Genes encoding beta-mannanases in *Brachypodium distachyon* seeds have a role not only in cell wall softening upon germination sensu stricto but also during post-germinative reserve mobilization

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Brachypodium distachyon seeds are characterized by presenting low levels of starch and high levels of (1,3; 1,4) β-glucans in its endosperm cells besides having thick mannan-rich cell walls. Upon germination and subsequent reserve mobilization β-mannanase encoding genes are selectively induced.
Endo-β-mannanases (EC 3.2.1.78) are hydrolytic enzymes that catalyze the cleavage of β(1-4) bonds in the mannan polymer. In the genome of Brachypodium, the endo-β-mannanase (MAN) family is represented by six members. We have systematically explored the expression of the six MAN genes in different organs (leaves, roots, spikes, developing seeds) and we have found that in dry seeds *BdMAN3* is the most highly expressed gene, but its expression decreases upon germination in aleurone while the other five are induced at 36 hours of imbibition. In the germinating embryo the most important genes are *BdMAN2* and *BdMAN6*. *In situ* hybridization analysis shows that *BdMAN*2 and *BdMAN6* transcripts accumulate in the coleorhiza while *BdMAN3* is mainly expressed in the radicle tip.
Difference in sequence and expression of the *MAN* gene family in *Brachypodium distachyon* and in other cereals, and their different putative functions in comparison to those reported in *Arabidopsis thaliana* [1,2] that are mainly involved in germination *sensu stricto,* are discussed.

References

[1] Iglesias-Fernández R, Rodríguez-Gacio MC, Carbonero P, Matilla AJ (2012) Softening-up mannan-rich cell walls. *J. Exp. Botany* 63: 3975-3988.

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